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# A multisyringe flow-through sequential extraction system for on-line monitoring of orthophosphate in soils and sediments

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## Abstract

A fully automated flow-through microcolumn fractionation system with on-line post-extraction derivatization is proposed for monitoring of orthophosphate in solid samples of environmental relevance. The system integrates dynamic sequential extraction using 1.0 mol l<sup>-1</sup> NH<sub>4</sub>Cl, 0.1 mol l<sup>-1</sup> NaOH and 0.5 mol l<sup>-1</sup> HCl as extractants according to the Hietjles-Lijklema (HL) scheme for fractionation of phosphorus associated with different geological phases, and on-line processing of the extracts via the molybdenum blue (MB) reaction by exploiting multisyringe flow injection as the interface between the solid containing microcolumn and the flow-through detector. The proposed flow assembly, capitalizing on the features of the multicommutation concept, implies several advantages as compared to fractionation analysis in the batch mode in terms of saving of extractants and MB reagents, shortening of the operational times from days to hours, highly temporal resolution of the leaching process, and the capability for immediate decision for stopping or proceeding with the ongoing extraction. Very importantly, accurate determination of the various orthophosphate pools is ensured by minimization of the hydrolysis of extracted organic phosphorus and condensed inorganic phosphates within the time frame of the assay. The potential of the novel system for accommodation of the harmonized protocol from the Standards, Measurement and Testing (SMT) Program of the Commission of the European Communities for inorganic phosphorus fractionation was also addressed. Under the optimized conditions, the lowest detectable concentration at the 3 $\sigma$  level was  $\leq 0.02$  mg P l<sup>-1</sup> for both the HL and SMT

schemes regardless of the extracting media. The repeatability of the MB assay was better than 2.5 % and the dynamic linear range extended up to 7.0 mg P l<sup>-1</sup> in NH<sub>4</sub>Cl and NaOH media and 15 mg P l<sup>-1</sup> whenever HCl is utilized as extractant for both the HL and SMT protocols.

*Keywords:* multisyringe flow injection, microcolumn sequential extraction, orthophosphate, soil, sediment

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## INTRODUCTION

Assessment of the bioavailability of macronutrients and trace elements in environmentally significant solid substrates is a key issue for ecology and environmental management. It is now widely accepted that the accessibility of the various elements for biota uptake depend strongly on their specific chemical forms and binding sites. A commonly used technique for identification of the phase associations of elements in solid phases is based on the application of sequential extractions [1-5]. These methods involve the rational use of a series of moderately selective reagents for releasing of targeted species from particular mineralogical fractions into the liquid phase under simulated natural and/or anthropogenic modifications of the environmental conditions. Sequential extraction procedures have been traditionally performed in a batchwise fashion. Yet, in the last decade, it has been realized that the conventional, manual procedures cannot mimic environmental scenarios accurately because naturally occurring processes are always dynamic, rather than static as they are identified by the traditional equilibrium-based approaches.

Recent trends have been focused on the development of alternative methods aimed at mimicking environmental events more correctly than the classical extraction counterparts [6]. Several attempts have been made on the characterization and evaluation of dynamic (non steady-state) partitioning methods, mostly exploiting continuous-flow or flow injection systems, where fresh portions of leaching agents are continuously provided to small containers or columns containing the solid material [7-18]. Dynamic approaches should be regarded as appealing avenues for fractionation assays not only because they alleviate the shortcomings of batch procedures including analyte re-adsorption and limited information on the size of actual available pools, but at the same time result also in improved precision and sample throughput. Furthermore, the

overall leaching process may be monitored as a function of the exposure time, giving rise to a more realistic insight into the extractability of elements from different geological reservoirs. As a consequence of the development of flow-based extraction approaches, on-line leachate measurements are readily applicable, as deduced from current trends in the field [9, 12-14, 16, 18, 19]. However, most of the works capitalize on hyphenated analytical methods based on coupling of the miniaturized column extraction manifold to continuously operating atomic spectrometers, such as flame atomic absorption spectrometry, inductively coupled plasma-mass spectrometry or inductively coupled plasma-atomic emission spectrometry, whereby the on-line generated extracts are directly injected into the detection system without any further treatment [9, 13, 14, 16, 18, 19].

As a result of the precise fluidic control via syringe pumps, the second generation of FI, namely, sequential injection (SI) analysis [20], has opened for new avenues in miniaturisation of sample processing including fractionation of solid samples [21]. While most FI-procedures employ continuous, uni-directional pumping of solutions, SI is based on exploiting programmable, bi-directional discontinuous flow as coordinated and controlled by a computer. Despite the well-recognized advantages of SI-microcolumn extraction as compared to its FI counterpart in terms of ruggedness, reagent consumption, precise handling of extracts and selection of the fractionation mode [6, 22, 23], automated post-column derivatization, which may be indispensable for macronutrient monitoring, is inherently hindered in SI due to the requirements of aspiration of the overall solutions into a holding coil [24].

To circumvent the above drawbacks, a hybrid flowing stream approach, the so called multisyringe flow injection (MSFI) analysis [25-27], is here proposed as the interface between the microcolumn system and detector for on-line microfluidic manipulation of leachates and reagents. To the best of our knowledge, MSFI, which compiles the advantageous features of FI and SI systems, has not been exploited for dynamic fractionation assays so far.

The hyphenated MSFI-microcolumn set-up has been assembled for automated flow-through partitioning and accurate determination of the content of bioavailable forms of orthophosphate in soils and sediments utilizing the Molybdenum Blue method for extract processing. Although environmental solids contain both organic and inorganic forms of phosphorus [28, 29], the latter are most relevant as a consequence of the well-known contribution to eutrophication [30]. In the terrestrial environment, phosphorus is an essential nutrient to support the plant growth; yet, direct uptake of organic forms is regarded to be unlikely.. For the very same reason it is essential that

the analytical approach used ensures that distinction between readily available inorganic phosphorus and organic bound phosphate reliably can be accomplished.

In this communication, the potential of the MSFI set-up for accommodation of two sequential extraction schemes involving different operationally defined extracting conditions is assessed. In this context, the Hietljes-Lijklema (HL) procedure [31] and the harmonized protocol from the Standards, Measurement and Testing (SMT) program of the European Commission [32] were selected and conducted in a dynamic fashion. Careful optimization of the chemical variables for the derivatization reactions is regarded to be crucial for appropriate performance of the analyzer due to the extreme pH conditions of the extracts obtained on-line in the various partitioning steps, not only to attain the desired selectivity, especially in regard to the presence of silicon, but also ensure the distinction between orthophosphate and organic-bound phosphorus.

## **EXPERIMENTAL**

### **Instrumentation**

The flow manifold devised for dynamic microcolumn fractionation and on-line spectrophotometric determination of orthophosphate is shown in Fig 1. It comprises a 5000-step syringe pump (SP) (Crison Instruments, Alella, Barcelona, Spain) for handling of the leaching reagents and performance of fractionation analysis; a ten-port multiposition selection valve (SV, Valco Instruments, Houston, TX) for selection of the appropriate extractant, and a multisyringe piston pump (MSP, MicroBu 2030, Crison Instruments) for on-line post-column derivatization of the extracts. The automatic SP is furnished with a 5 ml syringe (Hamilton, Switzerland) and a three-way solenoid valve at its head (SP1), which allows connection with the manifold or the carrier (water) reservoir. The central port of the SV is connected to SP via a holding coil (HC; used to house the selected extractant), which consists of a 1.42 m long PTFE tubing (1.5 mm i.d.), with an inner volume of 2.5 ml.

The MSP is equipped with four syringes (S1-S4) of 5, 2.5, 2.5 and 2.5 ml, respectively, whose pistons are connected in block to the same stepper motor. The solenoid commutation valves (V1-V4) (N-Research, Caldwell, NJ) placed at the head of the syringes permit the connection of the liquid drivers with the manifold (On) or with reagent reservoir (Off) regardless of the motion of the piston pump. This module also incorporates two additional discrete three-way solenoid valves (V5 and V6) for effecting soil extraction and on-line extract analysis under optimum experimental conditions.

All the connections including the extract loop and knotted reactors (KR) are made from PTFE tubing of 0.87 mm i.d. The length of the extractant loop, KR1 and KR2 are 55, 65 and 51 cm, respectively, corresponding to ca. 325, 385 and 300  $\mu\text{l}$ , respectively.

A diode-array spectrophotometer (Hewlett-Packard HP8452A) equipped with a flow-through cell (18  $\mu\text{l}$  inner volume, 1 cm optical path) is used as a detector. The analytical and reference wavelengths for monitoring of the phosphomolybdenum blue complex and minimization of Schlieren effects, respectively, are set at 690 and 530 nm. The transient spectrophotometric signals are acquired via an HP-IB interface at a frequency of 1.00 Hz. Instrumental control and data acquisition are performed using the software package AutoAnalysis 5.0 (Sciware, Spain).

### **Flow-through microcolumn assembly**

The design of the extraction microcolumn has been described in detail in a previous work [22]. Made of PEEK, it comprises a central dual-conical shaped sample container for facilitating fluidized-bed like mixing conditions. The entire unit is assembled with the aid of filter supports and caps at both ends. The membrane filters (Fluoropore<sup>TM</sup>, Millipore, 13 mm diameter with 0.45 and 1.0  $\mu\text{m}$  pore sizes for sediment and soil samples, respectively) used at both ends of the extraction microcolumn allowed solutions and leachates to flow freely through but retained the particulate matter. Solid amounts up to 300 mg can be automatically processed without clogging effects as reported elsewhere [19].

### **Reagents, solutions and samples**

All chemicals were of analytical-reagent grade and used as received. Solutions were prepared with double distilled water. A stock standard solution of orthophosphate (100 mg P  $\text{l}^{-1}$ ) was prepared from  $\text{KH}_2\text{PO}_4$  (Merck). Working solutions were prepared by stepwise dilution of the stock phosphorus solution. A stock standard solution of silicon (10 g Si  $\text{l}^{-1}$ ) was prepared from  $\text{Na}_2\text{SiO}_3 \cdot 5\text{H}_2\text{O}$ , and diluted standards were used for the investigation of the effect of silicate on the analytical readouts.

The chemical extractants used in both the HL and SMT sequential extraction schemes are summarized in Table 1 along with the geological phosphorus fractions released.

The reagent utilized in the HL scheme (R1) for post-column formation of molybdophosphoric acid was composed of 6 g  $\text{l}^{-1}$  ammonium molybdate (Panreac) in 0.3 M  $\text{H}_2\text{SO}_4$  (Merck) containing 0.125 % (w/v) oxalic acid (Probus). For the determination of HCl and NaOH extractable phosphorus in the SMT scheme, a solution of 6 g  $\text{l}^{-1}$  ammonium molybdate was

prepared in water (R3), and in 1.0 M H<sub>2</sub>SO<sub>4</sub> containing 0.25 % (w/v) oxalic acid (R1), respectively. A solution containing 0.15 g l<sup>-1</sup> SnCl<sub>2</sub> (Scharlau) and 0.94 g l<sup>-1</sup> hydrazine sulphate (Sigma) in 0.25 M H<sub>2</sub>SO<sub>4</sub> (R2) was employed for on-line reduction of the molybdophosphoric acid to the blue-coloured MB complex regardless of the fractionation scheme and extraction medium.

Two certified reference materials, namely SRM 2704 and SRM 2711, from the National Institute of Standards and Technology (NIST) were used for traceability studies. The SRM 2704 is a sediment collected from the Buffalo River in the area of the Ohio Street Bridge, NY, with a particle size distribution of 38-150 µm while the SRM 2711 is a pasture soil collected in the till layer of a wheat field (Montana, MT) with particle size < 74 µm. The conical microcolumn was packed, in both cases, with 50 mg solid samples, the estimated free column volume being 250 µl.

### **Dissolution of solid residues and samples**

Residues leftover after the sequential extraction schemes, and extracts collected downstream following post-column derivatization, were digested for quantitation of fixed and total released phosphorus, respectively. The microwave digestion procedure used can be regarded as a modified version of the EPA Method 3051 [33], named microwave-assisted acid digestion for sediments, sludges, soil suppress, and oils. Hence, digestions were performed in a closed-vessel microwave system (Milestone, model MLS-1200 Mega, Italy) using 1.0 ml of concentrated HNO<sub>3</sub> (65%, Scharlau) and 3.0 ml of concentrated HCl (30%, Scharlau). The microwave digestion program consists of 5 steps each lasting 5 min. The power program applied is detailed as follows: 250/400/650/250/0 W. After cooling, if needed, the digests were filtered through 0.45 µm cellulose acetate filters (Minisartfilters, Sartorius, Göttinger). The clear digests were made up to 50 ml and the content of orthophosphate was determined by spectrophotometry using a batchwise standard addition method.

The pseudo-total (aqua regia) phosphorus content in the NIST 2711 was determined using the microwave digestion conditions detailed above.

### **General procedure for flow-through sequential extraction**

The programmable flow-through fractionation assays were conducted with the aid of SP and SV. In the HL scheme, firstly, the HC was flushed with carrier (water), whereupon a 100 µl air plug from port 7 of SV and 2.0 ml of 1.0 M NH<sub>4</sub>Cl from port 1 were consecutively aspirated into HC. Afterward, V4 and V5 were turned 'On' and SP was set to dispense 250 µl of 1.0 M NH<sub>4</sub>Cl

(which matches the free column volume) from HC through the microcolumn at a flow rate of 3.0 ml min<sup>-1</sup>, allowing dynamic extraction to take place. The resulting leachate was stored into the extract loop and subsequently swept into the MSFI network for post-column derivatization and on-line determination of orthophosphate using a multicommutation protocol. The program was initially designed for 8 cycle runs (equivalent to 2.0 ml of extractant volume) but the operational sequence proceeds until quantitative stripping of labile phosphorus forms.

Prior to continuing with the ensuing HL extraction step, a washing protocol is implemented by aspiration of 100 µl of air and 2.0 ml of H<sub>2</sub>O from port 7 and 8, respectively, into HC, and using the same procedure described above for flow-through extraction. Thereafter, the next extractant (viz, 0.1 M NaOH or 0.5 M HCl) is aspirated repeatedly from the respective valve port and delivered to the soil containing microcolumn until completion of the phosphorus extraction. For the SMT protocol, the dynamic fractionation was performed using an identical operational sequence with 1.0 M NaOH and 1.0 M HCl as leaching reagents.

### **Multicommutation protocol for on-line post-column derivatization**

Two different multicommutation flow modalities for on-line injection of MB reagents, the so-called merging zones and sandwich-based approaches, were assayed. In both cases, switching of solenoid valves was effected during a single forward displacement of the piston bar of the MSFI pump. Both operational procedures are thoroughly described in the following:

*Merging zones mode:* As the name implies, the multicommutation protocol was programmed to merge the extract with the two reagent zones for development of the MB reaction as detailed in Table 2. After collection of the leachate in the extract loop, V1 and V2 were switched to ‘On’ while V5 and V6 were synchronously switched to ‘Off’. As a result, the orthophosphate zone merged with a well-defined plug of ammonium molybdate to yield the heteropolyacid species in KR1. Subsequently, V3 was activated to ‘On’, whereby the reaction zone reaching the next confluence point overlapped with the reducing SnCl<sub>2</sub> segment to form the blue-colored MB complex in KR2. The interdispersed zones were finally delivered downstream to the flow-through diode-array spectrophotometer by the carrier contained in S1, and the blue complex was monitored at 690 nm. Whenever the analysis was completed, all valves were returned to their original position for starting the following fractionation assay.



*Sandwich-based mode:* The multicommutation protocol involves the injection of two zones of molybdate which are stacked at each end of the leachate plug. The automated MSFI-multicommuted protocol using a sandwiched-based injection is summarized in Table 3. The method started when V2 was activated to ‘On’ and S2 was set to dispense 25 µl of molybdate into KR1. Thereafter, the combined extract/reagent zone was dispensed downstream. A second plug of molybdate (namely, 40 µl) was injected at the rear end of the leachate for sandwiching of the phosphorus containing segment. The reduction of molybdophosphoric acid to the molybdenum blue complex was performed in a merging zone fashion. To this end, 160 µl of SnCl<sub>2</sub> were injected at the front end of the interdispersed zone, and the transient signal of the MB complex was recorded by the detector.

## RESULTS AND DISCUSSION

### Configuration of the flow network for post-column phosphorus derivatization

#### *Implementation of the HL sequential extraction scheme*

In this three-step partitioning scheme, 1.0 M NH<sub>4</sub>Cl, 0.1 M NaOH and 0.5 M HCl are used as leaching reagents for consecutive extraction of phosphorus pools associated with different geological phases. The flow system was devised aimed at monitoring the orthophosphate, released on-line, via the MB method. Preliminary experiments were carried out for optimization of the MSFI configuration attending the variable chemical composition of the extracts, the volume of which was maintained at 250 µl. Two different reagent injection modalities, namely, the merging zones and the sandwich-type approaches, were assayed as described under Experimental. The merging zones was finally selected over the sandwich mode because the axial interdispersion between segments is not favored in a knotted coil [34,35], thus rendering double peak profiles.

The effect of MB reagent volumes (viz., ammonium molybdate and tin(II) chloride) on the analytical readouts was investigated taking into consideration the different sizes of the various syringes. The analytical sensitivity improved by 75% when increasing the size of the molybdate plugs from 40 to 80 µl, and remained constant up to 130 µl. This might be attributed to the compensation of the better radial mixing of the reagent and extract plugs with the higher dilution of the extract for volumes above 80 µl. Reagent volumes above 130 µl are unnecessary for the present design because they merely lead to undue dilution. Similar trends were obtained for the optimization of tin(II)chloride volume. To prevent excessive consumption of reagents, the

multicommutation protocol was programmed to merely inject 80 µl ammonium molybdate and 120 µl tin(II) chloride per assay.

The influence of the flow rate on the on-line MB derivatization reaction was evaluated over the range from 3.0 to 5.0 ml min<sup>-1</sup> (for S1) for the three extractant media of the HL scheme. The higher the flow rate the lower was the yield for MB formation, which is not surprising considering the relative slow reaction rate of the derivatization reaction. In fact, the peak height dropped by 20 % when increasing the flow rate from 3.0 ml min<sup>-1</sup> to 4.5 ml min<sup>-1</sup>. Yet, the higher the flow rate the lower is the yield of the competitive reaction for generation of the interfering molybdosilicate species and the better is the analytical throughput. Taking into account the variable sensitivity of the MB method in the various leachate solutions (see below) and the stripping of silicate from solid substrates in alkaline medium, the flow rate was fixed to 4.5 ml min<sup>-1</sup> for processing of the extracts obtained in the first two steps of the HL method. Regarding the apatite-phosphate fraction, it should be born in mind that the kinetics of formation of molybdophosphoric acid are not favoured in the acidic leachate medium. Therefore, a flow rate of 3.0 ml min<sup>-1</sup>, that can be programmed automatically, was utilized for monitoring of the orthophosphate released in the last step of the HL scheme.

### ***Implementation of the SMT sequential extraction scheme***

Within the framework of the Standards, Measurement and Testing Programme (SMT) of the European Commission, a batch extraction protocol for fractionation of phosphorus in environmental solids was harmonized in order to improve the reproducibility among laboratories [32]. The so-called SMT protocol was originally designed to obtain five phosphorus fractions, namely, total phosphorus (TP), inorganic phosphorus (IP), organic phosphorus (OP), apatite phosphorus (Ca-bound P) and non-apatite phosphorus (Al and Fe-bound P). It should be taken into account that some of the SMT partitioning steps are performed in a single rather than sequential manner and that the calcination of the solid residue as demanded for the TP and OP assays cannot be effected in an on-line fashion. Consequently, the potential implementation of the SMT fractionation assays related to the measurement of the inorganic (apatite + non-apatite) phosphorus fractions was ascertained. These two fractions are regarded as the most relevant ones for assessing the readily available phosphorus for plant uptake.

The SMT protocol is characterized for endorsing more aggressive leachants as compared with the HL scheme. The immediate consequence is that the optimal MSFI operational conditions for the HL fractionation as detailed above cannot be extrapolated directly to the SMT partitioning.

The use of 1.0 M rather than 0.1 M NaOH for leaching of phosphorus associated to hydrous oxides of Al and Fe (non-apatite phosphorus) facilitates the concomitant release of large amounts of silicate. Actually, a 20% increase in the analytical signals was detected whenever the molybdate reagent for HL containing 0.125 % (w/v) oxalic acid in 0.3 M H<sub>2</sub>SO<sub>4</sub> was utilized for analyzing a 1.0 mg P l<sup>-1</sup> standard containing 200 mg Si l<sup>-1</sup> in 1.0 M NaOH. It is known that the interference of silicate on the orthophosphate MB determination can be minimized by increasing both the acidity of the reaction medium and the concentration of the masking organic acid [36-39]. The effect of the concentration of sulfuric acid was thus evaluated from 0.3 to 1.5 M while that of oxalic acid from 0.125 to 0.25 (w/v). Yet, since the acidity has opposite effects on the selectivity and sensitivity of the molybdophosphate formation [36-38], the molybdate reagent was finally prepared in a 1.0 M H<sub>2</sub>SO<sub>4</sub> medium containing 0.25 (w/v) oxalic acid. Under these experimental conditions, silicate was tolerated up to 400 mg Si l<sup>-1</sup> at the 10% interference level.

As to the SMT apatite fraction, the method's sensitivity using the HL reagent decreased dramatically as a result of the slow development of the reaction for molybdophosphoric acid formation under strong acidic conditions. Therefore, the heteropolyacid forming reagent was prepared in distilled water and the reaction flow rate was affixed at 3.0 ml min<sup>-1</sup>. No appreciable increase of blank signals was detected, thus indicating that the acidity of the extractant suffices for preventing the self-reduction of molybdate. No oxalic acid was here added because of the negligible stripping of silicate from solids at low pH [40].

Although different reagents are needed for monitoring of the inorganic phosphorus in the HL and SMT extracts, a single MSFI assembly was arranged with no need for neither manual manipulations of reagents and leachates nor the changing of the composition of the carrier solutions for the various assays as demanded in previous flow systems for analyzing phosphorus containing soil solutions [40,41]. These facts emphasize the flexibility of MSFI for automated performance of derivatization reactions regardless of the variable chemical composition of the extracts in both fractionation schemes.

Figure 2 illustrates the extraction patterns as obtained by on-line MSFI analysis of minute volumes of leachate (namely, 250 µl) for SRM 2704 and SRM 2711 following microcolumn extraction using both the HL and the SMT schemes. The extractograms give rise to valuable knowledge on (i) the extraction kinetics, (ii) the content of phosphorus in available pools, (iii) the efficiency of the leachants, and (iv) the actual extractant volumes for quantitative release of orthophosphate. The SMT extractograms show sharp leaching profiles while those of HL depict a gradual stripping of the nutrient species as a result of the milder leachants used. This is especially

noticeable in the HCl extraction step in SRM 2704 where the amount of calcium bound phosphate leached in the first 2.0 ml in SMT is twofold higher to that of HL for the same extractant volume.

The analytical results for SRM 2704 and SRM 2711 also evidence that different types of samples, in this case, agricultural soil and river sediment, behave differently under the same extraction conditions (see extraction patterns for the Al and Fe-bound phosphate in Fig 2). This is attributed to the particular phosphorus soil phase associations existing in each kind of solid, thus, revealing the difficulty for setting a universal extraction protocol for dynamic fractionation of macronutrients.

### **Analytical performance of the MSFI analyzer**

Under the optimized chemical and physical variables, the dynamic linear range of the multicommutated MB method was established over the range 0.05-7.0 mg P l<sup>-1</sup> for the NH<sub>4</sub>Cl and NaOH extraction media in the HL scheme, and 0.35-15 mg P l<sup>-1</sup> for the 0.5 M HCl medium. For the NaOH and HCl steps in SMT, the regression lines extended from 0.1 to 7.0 and 0.5 to 15 mg P l<sup>-1</sup>, respectively. The limit of detection (LOD) assessed from three times the standard deviation ( $\sigma$ ) of either the blank or a 50  $\mu$ g P l<sup>-1</sup> standard solution were 0.01, 0.01 and 0.02 mg P l<sup>-1</sup> for NH<sub>4</sub>Cl, NaOH and HCl steps in HL, respectively. In SMT, LODs of 0.02 and 0.01 mg P l<sup>-1</sup> for 1.0 M NaOH and 1.0 M HCl were, respectively, obtained. Repeatability was estimated from 10 consecutive injections of a 1.0 mg P l<sup>-1</sup> standard solution in each extracting medium. Relative standard deviations (RSD) of 1.0, 0.9 and 2.4% were obtained for the NH<sub>4</sub>Cl, NaOH and HCl solutions in HL, respectively, and 1.0 and 2.2 % for the NaOH and HCl media in SMT, respectively.

In Table 4, the analytical performance of the on-line MSFI analyzer for HL fractionation and orthophosphate determination is critically compared with that of the batchwise method [31] and an SI microcolumn extraction (SI-MCE) system with further off-line analysis of the extracts by flow injection analysis [42]. A distinguishing feature of the MSFI-multicommutation set-up with respect to the FI and batch systems is the minimum consumption of reagents. Thus, in the proposed system, the consumption of MB chemicals is reduced more than 15-fold as regards to former methods. This is a consequence of the time-based injection of well-defined volumes of solutions at the precise instant for development of the reactions as effected via activation of the solenoid valves. Whenever not needed, the MB reagents are delivered to their respective reservoirs in lieu of being propelled downstream as occurs in continuous, forward-flow

assemblies. Therefore, discontinuous-flow based MSFI analyzers might be viewed as environmentally friendly chemical processors.

The slightly higher operational time in MSFIA versus SI-MCE/FIA for fractionation/determination of orthophosphate has its origin in the different number of extracts analysed, that is, 86 extracts in the proposed on-line system as compared with merely 27 using the former FI set-up. Yet, the potential of the flow-through hyphenated MSFI system for handling of minute fractions of extract, that is  $\leq 250 \mu\text{l}$  versus  $\geq 5000 \mu\text{l}$  for methods involving fraction collection, ensures a unrivaled temporal resolution that yields a detailed insight into the leaching kinetics of phosphorus from the different soil/sediment compartments, as illustrated in Fig 3. Hence, the content of phosphorus in readily mobilisable reservoirs can be more accurately estimated. At the same time, the sample mass to extractant volume ratio for each sequential extraction step can be calculated with improved accuracy, thereby avoiding the usage of any surplus of reagent for quantitative leaching of phosphorus in the various geological phases. According to Fig 3 and data presented in Table 4, the leachant volumes used in the MSFI on-line fractionation system are three to four times lower to those required whenever off-line measurements are applied.

#### **Accuracy of the proposed flow-through microcolumn extraction-multisyringe flow injection system for orthophosphate fractionation**

The SRM 2704 river sediment and SRM 2711 Montana soil were utilized to evaluate the reliability and accuracy of the developed flow-through microcolumn hyphenated method. The amount of extractable orthophosphate in both standard reference materials resulting from the application of dynamic partitioning is listed in Tables 5 and 6 along with the certified total phosphorus content and the pseudototal (aqua regia) phosphorus concentrations. The amount of extractable phosphorus for both solid substrates determined on-line by summation of all fractions plus residue is much lower than the certified values, while a good agreement, with maximum deviations of 8 %, is obtained between the summation of fractions for SRM 2704 and SRM 2711 following acidic microwave digestion of the extracts collected downstream (after on-line detection) and the pseudototal and endorsed concentrations, respectively.

According to the MB chemistry only dissolved orthophosphate can react with MB reagents for generation of the reduced blue-colored complex. Therefore, the on-line MSFI extraction system with spectrophotometric monitoring detects merely the extractable, reactive inorganic phosphorus. However, in the off-line assays, the extracts that may contain other phosphorus

compounds, such as organic phosphorus and condensed inorganic phosphates (see below), can be degraded under microwave digestion into orthophosphate, thus providing information on the total phosphorus released. As a result, the total phosphorus content after appropriate processing of the MSFI extracts is not significantly different at the 0.05 significance level to the certified phosphorus concentrations in both SRM materials, as shown in Tables 5 and 6.

### **Comparison of on-line and off-line modes for phosphorus determination in soil/sediment extracts**

The phosphorus fractionation results using the on-line hyphenated MSFI assembly for SRM 2711 were contrasted with those previously obtained by sequential injection microcolumn extraction (SI-MCE) and fraction collection prior to off-line FI analysis [42]. As shown in Table 5, the extractable phosphorus monitored spectrophotometrically is severely affected by the mode of extract processing. It should be stressed that even though the HL and SMT schemes were originally designed for inorganic phosphorus fractionation, soil organic phosphorus can be concurrently released by the extracting reagents themselves. In fact, the most commonly accepted schemes for soil organic phosphorus fractionation [43,44] involve alkaline and acid extractions with 0.1-0.5 M NaOH and  $> 0.1$  M HCl, respectively, thus matching the chemical conditions for HL and SMT extractions. NaOH creates electrostatic repulsions by increasing the negative charge of both organic and mineral components [45] while HCl dissolves salts of some organic phosphate esters that are relatively insoluble in alkaline solution [43].

Furthermore, several organic phosphates are instable in alkaline and acid conditions, and therefore might be hydrolyzed under the fractionation conditions to free phosphate, thus leading to the overestimation of the content of readily accessible orthophosphate. The effects of alkaline and acid hydrolysis for a wide range of soil organic phosphorus compounds have been found to be markedly influenced by the nature of the phosphorus species [44,46,47]. The extraction of fast hydrolysable phosphorus forms (e.g., phosphatidyl choline) may hence explain the discrepancy in Table 5 between the on-line and off-line (SI-MCE) data for NaOH and HCl extracts in the HL scheme. Regarding the  $\text{NH}_4\text{Cl}$  extracts in HL, the contribution from organic phosphorus hydrolysis should here be negligible because organic substances cannot be leached under mild extraction media. Yet, common sources of readily hydrolysable phosphorus in soils, that might be released in the first extraction step of HL, are the condensed inorganic phosphates (e.g., pyrophosphate and polyphosphates) [44].

It should be noted that the hydrolysis of organic phosphorus and condensed inorganic phosphates in off-line based fractionation analysis might occur not only during the timeframe of the extraction but to a significant extent during the residence period prior to actual phosphorus determination. As opposed to batch partitioning and dynamic methods involving off-line or at-line analysis of phosphorus containing fractions, the novel multisyringe flow approach leads to a substantial shortening of the assay protocol, thus minimizing the potential decomposition of hydrolyzable phosphorus compounds. Each microvolume of extract leaving the microcolumn is readily treated on-line with MB reagents, while the released organic species in the batchwise method and SI-MCE system with off-line analysis remain in intimate contact with the extracting reagent for > 15 and 3 h, respectively. Therefore, the flow-through MSFI fractionation analyzer with MB detection should be regarded as a unique tool for accurate monitoring of mobilisable orthophosphate in environmental solid substrates, even though organic phosphorus may be leached with the extracting reagents.

## CONCLUSION

Multisyringe flow injection analysis has been presented as an appealing analytical tool for on-line processing of the extracts generated in flow-through dynamic fractionation assays. Prominent features of the assembled analytical set-up involving multicommutated post-column injection of reagents are the considerable saving of chemicals, the high temporal resolution of the leaching processes, the accurate evaluation of sample mass to leachant volume ratios, and the improved reliability, robustness, and automation with respect to methods with off-line analysis of extracts. The intrinsic versatility of the MSFI analyzer has been exploited for the accommodation in a single set-up of two well-accepted schemes for fractionation of inorganic phosphorus, *i.e.* the HL and SMT protocols, even though the chemical composition of the extracts resulting from both schemes is significantly different. As a consequence of the most aggressive extractants utilized in the SMT protocol, higher leachable contents and sharper extraction profiles were encountered as compared to the HL scheme. However, SMT does not give information on the labile inorganic phosphorus, which is regarded to be the readily available fraction for plant uptake. Furthermore, the extreme chemical conditions adopted in this scheme are unlikely to mimic the changes in the chemical properties of the solid occurring under environmental scenarios.

The flow-through microcolumn system hyphenated with spectrophotometric detection has been also proven unique for accurate monitoring of available orthophosphate in the extracts due



to the minimization of the potential hydrolysis of extracted organic and condensed phosphorus into orthophosphate.

Further research is aimed at expanding the flexibility of the multisyringe flow injection analysis system for monitoring of ultratrace levels of pollutants, viz., heavy metals and metalloids, in solid substrates following dynamic fractionation and on-line solid-phase extraction for isolation and preconcentration of the targeted species.

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## FIGURE CAPTIONS

**Figure 1.** Schematic diagram of the hybrid flow-through microcolumn extraction/MSFI system for automated fractionation and on-line determination of orthophosphate. R1: 6 g l<sup>-1</sup> ammonium molybdate + 0.125 % (w/v) oxalic acid in 0.3 M H<sub>2</sub>SO<sub>4</sub> (for HL scheme) or 6 g l<sup>-1</sup> ammonium molybdate + 0.25 % (w/v) oxalic acid in 1.0 M H<sub>2</sub>SO<sub>4</sub> (for NaOH-P in SMT). R2: 0.15 g l<sup>-1</sup> SnCl<sub>2</sub> + 0.94 g l<sup>-1</sup> N<sub>2</sub>H<sub>4</sub>·H<sub>2</sub>SO<sub>4</sub> in 0.25 M H<sub>2</sub>SO<sub>4</sub> and R3: 6 g l<sup>-1</sup> ammonium molybdate in water (for HCl-P in SMT). SP: Syringe pump; MSP: Multisyringe pump; SV: Selection valve, V: Solenoid valve, KR: Knotted reactor, D: Detector.

**Figure 2.** Comparison of the on-line MSFI extraction profiles of orthophosphate in (a) SRM 2704 and (b) SRM 2711 as obtained by the application of HL and SMT protocols in a dynamic fashion. The labile phosphorus in 1.0 M NH<sub>4</sub>Cl was < LOD for SRM 2704. The concentration of orthophosphate is given as mg P/kg sample.

**Figure 3.** Comparison of orthophosphate extractograms for the HCl step in the HL scheme for SRM 2711 as obtained by using off-line MB detection (5 ml per subfraction) and on-line MSFI spectrophotometric analysis (250 µl per subfraction).

**Table 1.** Leaching reagents and corresponding phosphorus fractions in two extraction procedures

Procedure	Step I	Step II	Step III
<b>Hieltjes-Lijklema</b>	1.0 M $\text{NH}_4\text{Cl}$ , pH 7 (Labile P)	0.1 M NaOH (Fe and Al-bound P)	0.5 M HCl (Ca-bound P)
<b>SMT</b>	1.0 M NaOH (Fe and Al-bound P)	1.0 M HCl (Ca-bound P)	

**Table 2.** Multicommuted merging zone protocol for on-line post-column derivatization

Multicommutation step	Solenoid valve position					Reagent ( $\mu\text{l}$ )		Carrier ( $\mu\text{l}$ )	Total flow rate ( $\text{ml min}^{-1}$ )
	V1	V2	V3	V4	V5	R1	R2		
Merging of the extract and R1	On	On	Off	Off	Off	80	-	160	6.75
Delivery of the reaction zone to KR2	On	Off	Off	Off	Off	-	-	160	4.50
Merging of the reaction zone with R2	On	Off	On	Off	Off	-	120	240	6.75
Delivery of the MB zone to detector	On	Off	Off	Off	Off	-	-	2600	4.50

**Table 3.** Multicommutated sandwich-type protocol for on-line post-column derivatization

Multicommutation step	Solenoid valve position					Reagent ( $\mu\text{l}$ )		Carrier ( $\mu\text{l}$ )	Total flow rate
	V1	V2	V3	V4	V5	R1	R2	(S1)	( $\text{ml min}^{-1}$ )
Fronting zone of R1	Off	On	Off	Off	Off	25	-	-	2.25
Simultaneous delivery of extract and R1	On	On	Off	Off	Off	80	-	160	6.75
Delivery of the rear end of extract into MSFI network	On	Off	Off	Off	Off	-	-	90	4.50
Tailing zone of R1	Off	On	Off	Off	Off	40	-	-	2.25
Injection of R2	On	Off	On	Off	Off	-	160	320	6.75
Delivery of the MB zone to detector	On	Off	Off	Off	Off	-	-	2440	4.50

**Table 4.** Comparison of the extraction/determination parameters for the HL scheme in SRM 2711 for three different procedures applied to phosphorus fractionation and extract analysis

Parameters	MSFIA (this work)		SI-MCE/FIA [43]		Batch [32]	
	Amount(mg)	Volume( $\mu$ l)	Amount(mg)	Volume( $\mu$ l)	Amount(mg)	Volume( $\mu$ l)
1. Reagent consumption per extract analysis:						
Ammonium molybdate	0.5	80	7.2	600	6.0	150
Reducing agent <sup>a</sup>	0.02	120	0.2	600	5.3	300
Masking agent <sup>b</sup>	0.1	80	2.4	1000	0.1	300
2. Extractant volume (ml)						
NH <sub>4</sub> Cl fraction	4.5		25		2x50	
NaOH fraction	6		30		50	
HCl fraction	11		30		50	
3. Analysis time per extract (min)	0.77		0.74		20	
4. Operational time for overall fractionation						
<del>and extract analysis per sample (h)</del>	<del>2.8</del>		<del>2.7</del>		<del>45.3</del>	

<sup>a</sup> Tin(II) chloride was used as a reducing reagent for MSFIA and SI-MCE/FIA, while ascorbic acid was employed for batchwise analysis.

<sup>b</sup> Oxalic acid was employed as a masking agent for MSFIA, and tartaric acid for the SI-MCE/FIA and batch approaches.

**Table 5.** Comparison of extractable amounts of phosphorus in SRM 2711 ( $\text{mg kg}^{-1}$ ) as obtained by using the Hieltjes-Lijklema and SMT sequential extraction schemes with on-line and off-line MB detection modes

Fractions	Hieltjes-Lijklema			SMT	
	On-line (MSFIA)	Off-line <sup>a</sup> (MWD)	Off-line <sup>b</sup> (SI-MCE) [43]	On-line (MSFIA)	Off-line <sup>a</sup> (MWD)
(1) Labile P	$7 \pm 1$	$36 \pm 2$	$45 \pm 5$		
(2) Al and Fe-bound P	$13.1 \pm 0.4$	$84 \pm 6$	$93 \pm 10$	$11 \pm 1$	$105 \pm 2$
(3) Ca-bound P	$324 \pm 45$	$370 \pm 25$	$373 \pm 18$	$365 \pm 9$	$384 \pm 9$
(4) Residue	$215 \pm 14$	$215 \pm 14$	$288 \pm 43$	$202 \pm 9$	$202 \pm 9$
Summation (1+2+3+4)	$559 \pm 47$	$705 \pm 29$	$799 \pm 48$	$578 \pm 13$	$690 \pm 13$

Results are expressed as the mean of 5 fractionation assays  $\pm$  SD

Pseudo total P:  $700 \pm 30 \text{ mg P kg}^{-1}$ ; Certified value:  $860 \pm 70 \text{ mg P kg}^{-1}$

MWD: Microwave digestion; SI-MCE: Sequential injection-microcolumn extraction

<sup>a</sup>The concentration of orthophosphate in the extracts is determined by batchwise MB standard addition

<sup>b</sup>The concentration of orthophosphate in the extracts is determined by FIA-MB



**Table 6.** Comparison of extractable amounts of phosphorus in SRM 2704 (mg kg<sup>-1</sup>) as obtained by using the Hieltjes-Lijklema and SMT sequential extraction schemes with on-line and off-line MB detection modes

Fractions	Hieltjes-Lijklema		SMT	
	On-line (MSFIA)	Off-line <sup>a</sup> (MWD)	On-line (MSFIA)	Off-line <sup>a</sup> (MWD)
(1) Labile P	< LOD	< LOD		
(2) Al and Fe-bound P	114 ± 14	103 ± 6	164 ± 18	177 ± 14
(3) Ca-bound P	310 ± 43	595 ± 17	288 ± 18	664 ± 21
(4) Residue	232 ± 26	232 ± 26	171 ± 5	171 ± 5
Summation (1+2+3+4)	656 ± 52	930 ± 32	623 ± 26	1012 ± 26

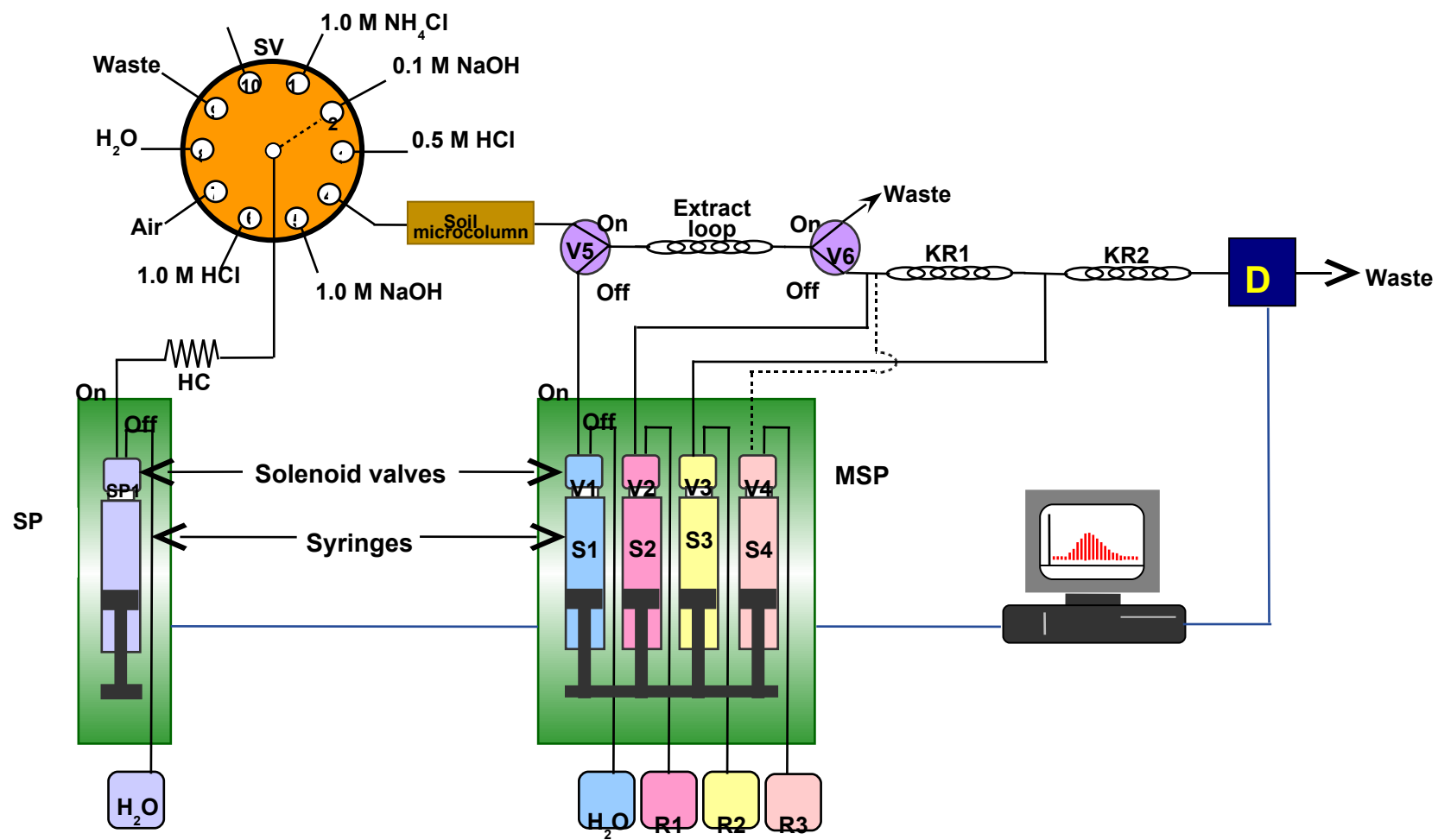
Results are expressed as the mean of 5 fractionation assays ±SD

Certified value: 998 ± 28 mg P kg<sup>-1</sup>

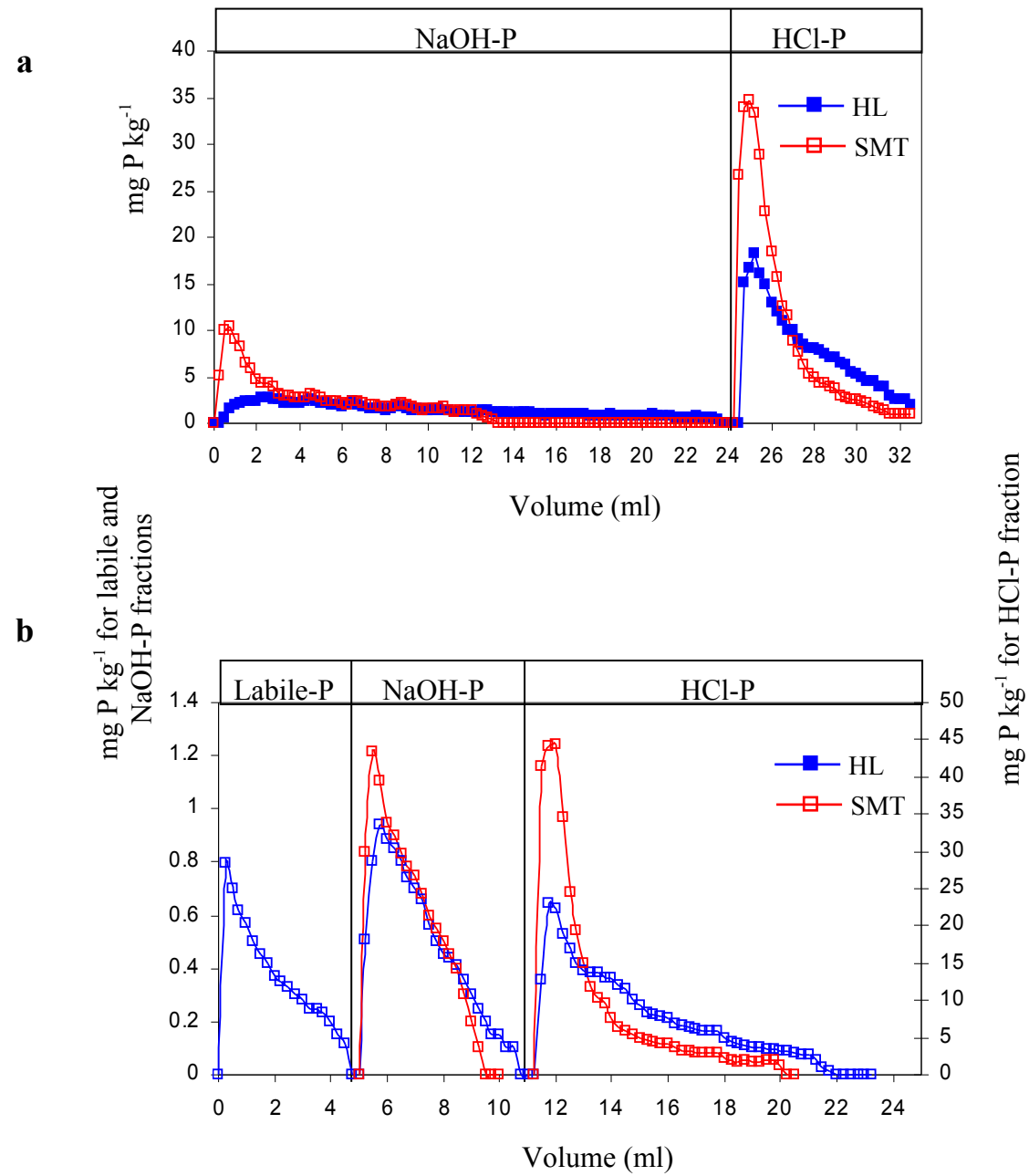
LOD: Limit of detection

<sup>a</sup>The concentration of orthophosphate in the extracts is determined by batchwise MB standard addition.

FIGURE 1



**FIGURE 2**



n-line mode

FIGURE 3

mg P

